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THE INFLUENCE OF EXTRACTS OF ANCHYLOSTOMA CANINUM ON THE COAGULATION OF THE BLOOD AND ON HEMOLYSIS.*

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IN former experiments, one of us (Loeb, with A. J. Smith) had found that in the anterior part of *Anchylostoma caninum* a substance is present that has a distinctly inhibiting action upon the coagulation of the blood.¹ At that time we did not carry out experiments concerning the hemolytic action of such extracts, but we noticed that in the mixtures used for the determination of the coagulation-time no distinct hemolytic influence of the extract could be observed.

In the posterior part of *anchylostoma* little of the substance inhibiting the coagulation was present in one experiment, while in the other experiments it was absent. Boiling the extract during a period of 15 minutes caused a marked weakening of its activity, without, however, destroying it entirely.

At that time Loeb had assumed that the substance present in *anchylostomum* would in all probability be found to be similar to hirudin, the substance of the leech which inhibits coagulation, in fact the analogy which at that time appeared to exist between the behavior of the leech and of *anchylostomum* induced one of us to undertake these experiments.

In order to test this hypothesis we carried out new experiments. For some time past we searched for specimens of *Anchylostoma caninum* in all the dogs upon which we performed an autopsy. The *anchylostoma* found were carefully washed and afterward dried with filter-paper. The worms thus freed from adhering intestinal contents were ground up in a porcelain mortar and the ground up material was dried at a temperature varying between approximately 42° and 50° C. The dried material was scraped off and collected in a bottle for further use. In this way approximately 170 mgm. dried substance of *Anchylostoma caninum* were obtained, which enabled us to carry out a limited number of experiments.

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¹ *Centralbl. f. Bakt.*, 1904, Abt. 1, Orig., 37, p. 93; *ibid.*, 1906, Abt. 1, Orig., 40, p. 738.

THE INFLUENCE OF ANCHYLOSTOMA EXTRACT ON COAGULATION
OF THE BLOOD.

Before giving our results, it might be advisable to state briefly the reactions that are characteristic of hirudin. If a moderate quantity of hirudin is added to blood *in vitro*, the plasma obtained after centrifugation coagulates readily under the influence of the tissue-coagulins as they exist in the extracts of the liver and kidney and it also coagulates after the addition of a suspension of mammalian erythrocytes that have previously been hemolyzed. Usually tissue extracts accelerate the coagulation to a high degree if a certain optimal quantity is added; a still larger quantity of tissue extract acts less favorably.

Blood serum has relatively a very slight coagulating effect upon hirudinplasma; but the larger the quantity of blood serum added, the more marked is the accelerating effect upon the coagulation of hirudinplasma. A combination of blood serum and tissue extract acts in a similar manner as tissue extract alone or slightly better, if it is used immediately after the substances have been mixt. If, on the other hand, the combination is used approximately after 10 minutes' standing, the serum exerts a markedly inhibiting action upon the coagulating effect of the tissue extract.

A combination of a suspension of hemolyzed erythrocytes and of blood-serum coagulates more strongly than either alone, especially, if the mixture has been standing for 15 minutes before it is used.

If we now study the action of these various substances upon blood plasma to which a certain amount of anchylostoma extract had been added, we find that the "anchylostomaplasma" obtained by centrifugation of the blood differs in its reactions markedly from the hirudinplasma.

Extract of kidney which exerts a very active coagulating effect upon hirudinplasma is without such influence if added to anchylostomaplasma and it may even inhibit the action of other coagulating agencies. Blood serum, on the other hand, which accelerates the coagulation of the hirudinplasma only to a very slight extent has a very marked coagulating influence upon anchylostomaplasma.

In a combination of serum and kidney extract the kidney extract seems even to possess an inhibiting influence upon the coagulating

power of the serum if both are added to anchylostomaplasma. Hemolyzed dog-erythrocytes, however, exert a pronounced coagulating power upon hirudin as well as upon anchylostomaplasma and a combination of serum and erythrocytes which had been kept at room temperature for 10 minutes before being added to the anchylostomaplasma was extremely active.

The addition of various quantities of CaCl_2 to the anchylostomaplasma either alone or in combination with kidney extract did not cause coagulation. (0.1 and 0.2 c.c. of a 1.22 per cent CaCl_2 solution were added to 1 c.c. of anchylostomaplasma or to a mixture of 2 c.c. containing 1 c.c. anchylostomaplasma and various quantities of kidney extract and 0.85 per cent NaCl solution.)

Addition of an equal amount of distilled water to the anchylostomaplasma caused a very slow coagulation. Three hours after the addition of the water no trace of coagulation was noticeable. Six hours after the addition of the distilled water the coagulation was not quite complete. It must, however, be stated that the distilled water had been added on the day following the preparation of the plasma.

In regard to the quantities of anchylostoma-powder needed, the following observations were made: In one experiment 85 mgm. were suspended in 6 c.c. of a 0.85 per cent NaCl solution. In this mixture, which reacted approximately neutral to litmus-paper 14 c.c. dog-blood were received with the aid of a canula introduced in a femoral artery. The plasma obtained from this mixture remained entirely liquid for 24 hours; but it would probably have remained still longer in a liquid state, if some substances causing coagulation had not been added at various periods. If we consider that the powder represents the substance of the whole body of the anchylostoma, while the active substance is produced only in the anterior part of the body (probably in one of the glands), furthermore, that the largest part of the powder is entirely insoluble in water or in blood plasma and that the active substance can have represented only a very small fraction of the powder, we are justified in the conclusion that the substance exerts a strong anticoagulating influence.

In another experiment 13 mgm. of anchylostoma-powder were suspended in 5 c.c. NaCl solution. In this case the control-blood collected in an equal amount of 0.85 per cent NaCl solution coagu-

lated in $2-2\frac{1}{2}$ minutes, while 2 c.c. blood mixt with 2 c.c. of the anchylostoma suspension coagulated in 39 minutes. In this case the quantity of anchylostoma used was much smaller and the erythrocytes which remained in contact with the plasma exerted a coagulating influence upon the fibrinogen.

A part of the protocol of Experiment 2 may be given as an example:

D. A. P. = Dog-plasma + anchylostoma extract
 D. X. = Dog-kidney extract
 D. S. = Dog-blood serum
 D. C. P. = Dog-erythrocytes

1 c.c. D. A. P. +	1 c.c. D. X.	not yet coagulated	22 hours
1 c.c. D. A. P. + 0.5 "	D. X. " " "	"	22 "
1 c.c. D. A. P. + 0.3 "	D. X. " " "	"	22 "
1 c.c. D. A. P. control	" " "	"	22 "
1 c.c. D. A. P. + 2 c.c.	D. S.	coagulated	35 minutes
1 c.c. D. A. P. + 0.7 "	D. S. "	"	1 hour 20 minutes
1 c.c. D. A. P. + 0.3 "	D. S. "	4 "	20 "
1 c.c. D. A. P. + 0.3 c.c.	D. C. P. "	1 "	38 "
1 c.c. D. A. P. + [0.7 c.c. D. S. + 0.3 c.c. D. X. being mixt and added immediately to D. A. P.] coagulated 2 hours 58 minutes.			
1 c.c. D. A. P. + [0.7 c.c. D. S. + 0.3 c.c. D. C. P. 10 minutes after mixing added to D. A. P.] coagulated 1 minute 50 seconds.			

We may summarize as follows the principal facts which we found in our study of the action of anchylostoma upon the coagulation of the blood:

1. In *Anchylostoma caninum* a substance is produced inhibiting the coagulation of the blood *in vitro*.
2. This substance is formed in the anterior part of the body of anchylostoma, presumably in one of its glands.
3. This substance is not destroyed, but markedly weakened by boiling during a period of 15 minutes.
4. It can be preserved for a long time by drying the anchylostomas and then converting them into a powder.
5. After the addition of a sufficient quantity of anchylostoma extract the blood-plasma does not, or only with great difficulty, coagulate after the addition of tissue extract; it coagulates relatively readily after the addition of blood-serum and of hemolyzed erythrocytes.
6. A combination of erythrocytes and serum proved to be extremely active.

7. Addition of CaCl_2 to the plasma alone or to a combination of plasma and tissue extract does not cause coagulation.

8. On the second day dilution with distilled water led to a very slow coagulation of the plasma, but in this case the possibility could not be excluded that at that time the plasma was near the point of spontaneous coagulation; lack of material prevented us from carrying out desirable control tests in this case.

From these facts we can draw the conclusion that the inhibiting substance in anchylostoma is not analogous to hirudin. We have at present no indication that the inhibiting substance acts by inactivating calcium; in consideration of the small quantities of the substance needed for obtaining an anticoagulating effect, we can exclude the hypothesis that the action is due to the presence of sufficient quantities of the common inorganic salts which, in themselves, exert a certain inhibiting influence.

Altho at the present time we cannot make any positive statement concerning the nature of this substance, we wish to draw attention to a certain similarity which seems to exist between the action of cobra-venom and anchylostoma extract upon the coagulation of the blood. According to Morawitz¹ after the addition of cobra-venom, the plasma also coagulates relatively more easily under the influence of serum than of tissue extract.

Weinberg² found that *Sclerostomum equinum* in many respects behaves in a manner similar to anchylostoma and this investigator found also that extract of *Sclerostomum equinum* inhibits markedly the coagulation of the blood. It would be of interest to examine the character of the anticoagulating substance of sclerostomum and to compare it with that of anchylostoma. The former may also be found not to resemble hirudin.

DOES ANCHYLOSTOMA EXTRACT CAUSE HEMOLYSIS?

In our previous experiments in which we made use of the extracts of fresh anchylostoma we found no noticeable hemolysis in our blood-anchylostoma extract mixture. At that time, however, we had been mainly concerned with the effect of the extracts upon the coagulation

¹ *Deutsches Archiv f. klin. Med.*, 1904, 80, p. 340.

² *Ann. de l'Inst. Pasteur*, 1907, 21, pp. 417, 531.

of the blood and not upon hemolysis; and we had not carried out experiments in which we used washed erythrocytes.

In this series we carried out some additional experiments in which we tested the extracts of anchylostoma powder (which we had obtained in the manner indicated above) upon erythrocytes of the dog, freed from serum through washing with 0.85 per cent NaCl and by centrifugalizing and by repeating this process four times.

The results of these experiments can be briefly stated. We added 5 mgm. or less of the anchylostoma powder to 1 c.c. of a 5 per cent suspension of washed dog-erythrocytes. This quantity of powder did not exert the slightest hemolytic effect upon the erythrocytes of the dog. After this fact had been established we tested combinations of lecithin and anchylostoma extract. Lecithin when used in doses in which it alone did not exert a hemolytic effect did not activate anchylostoma extracts.

The anchylostoma extract contained 5 mgm. of anchylostoma powder in each c.c. of 0.85 per cent NaCl solution, and the lecithin solution was prepared by diluting a 1 per cent solution of Kahlbaum's lecithin in methylalcohol 100 times with a 0.85 per cent NaCl solution. As much as 1 c.c. of the anchylostoma extract was used in combination with 2.5 c.c. and with smaller quantities of the lecithin suspension, without producing any more hemolysis than in the control, in which lecithin alone had been used or in which merely 0.85 per cent NaCl solution had been added to the erythrocytes. The same quantity of anchylostoma powder exerts an extremely strong inhibiting influence on the coagulation of 1 c.c. blood-plasma.

In interpreting these results we have to admit the possibility that the drying of the anchylostoma substance at a temperature varying between 42° and 50° C. may have destroyed a hemolysin which might possibly have been present. This is, however, a not very probable assumption, if we consider that the degree of heat increase was slight and that the amount of water in the anchylostoma powder was reduced to a minimum within a very short time.

Weinberg found in sclerostomum a hemolytic substance, soluble in NaCl solution and resistant to heating to 115–20° C. during a period of 15–20 minutes. It is not very probable that in our case the drying process which took place between 42° and 50° should have destroyed

the hemolysin. Whipple¹ believes that only a very weak hemolysin is present in anchylostoma extracts; he did not observe a hemolytic action if less than 20 anchylostoma were extracted in 1 c.c. of 0.852 per cent NaCl. Our experiments and those of Whipple prove that the hemolytic action of anchylostoma must be very weak indeed, if at all present. Preti found² in anchylostoma a hemolytic substance that was not soluble in a physiological NaCl solution, but was soluble in ether and alcohol; it was heat-resisting. Preti evidently did not observe a hemolytic effect of the extract made by means of a 0.85 per cent NaCl solution. The etiological significance of the hemolytic action of anchylostoma appears to be very doubtful, especially if we consider that certain normal organs as well as the contents of the small intestines contain hemolytic substances without causing anemia. If hemolysis plays any part in the anemia of uncinariasis, it might as well be caused by an absorption of certain parts of the contents of the small intestines through the injured intestinal wall as by the very weak hemolytic action of anchylostoma.

However that may be, we can be certain that the anchylostoma contains a substance inhibiting the coagulation of the blood and that this substance favors probably the continued bleeding following an injury of the intestinal wall; and that the presence of such a substance is the more important because the intestinal mucosa would exert a very marked coagulating action upon the blood and no after-bleeding would take place, without the existence of a special anti-coagulating substance.

CONCLUSIONS.

1. In the anterior part of anchylostoma a substance is present that inhibits the coagulation of the blood; it can be preserved for a long time in a dried condition. It is not analogous to hirudin, but it seems to show some similarity to the substance inhibiting the coagulation of the blood which is present in cobra-venom. It will, however, be necessary to make additional comparative tests before such a relationship can be considered proven.

2. The powder of anchylostoma which we used did not contain a direct hemolytic substance, nor a substance which can be activated through a combination with lecithin.

¹ *Jour. Exper. Med.*, 1909, 11, p. 331.

² *Münch. med. Wchnschr.*, 1908, 55, p. 436.